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A Mechanism of Haloalkene-Induced Renal Carcinogenesis

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Several halogenated alkenes are nephrotoxic; some others induce renal tubular adenocarcinomas in rodents after lifelong administration. A bioactivation mechanism accounting for the organ-selective tumor induction has been elucidated: conjugation of the parent compounds with glutathione (GSH), catalyzed by hepatic GSH *S*-transferases, results in the formation of haloalkyl and halovinyl glutathione *S*-conjugates. Formation of *S*-conjugates (identified by NMR and mass spectrometry) could be demonstrated with trichloroethene, tetrachloroethene, hexachlorobutadiene, perfluoropropene, trichlorotrifluoropropene, and dichloroacetylene in incubations with rat liver microsomes and in the isolated perfused rat liver. The GSH conjugates formed are eliminated from the rat liver with the bile and may be translocated to the kidney, intact or after metabolism to the corresponding cysteine *S*-conjugates that are metabolized in the kidney by renal tubular cysteine conjugate β-lyase (β-lyase) to reactive intermediates, most likely thioacylchlorides and thioketenes. Interaction of these potent electrophiles with DNA [demonstrated for intermediates formed from *S*-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine] causes mutagenicity in bacteria, genotoxicity in cultured renal cells, and cytotoxicity in kidney cells. As an alternative to β-lyase-catalyzed cleavage, the cysteine *S*-conjugates may be acetylated to the corresponding mercapturic acids, which have been identified in urine. The ability of the kidney to concentrate GSH and cysteine *S*-conjugates and the intensive metabolism of GSH *S*-conjugates to cysteine *S*-conjugates in this organ are evidently responsible for the organotropic carcinogenicity.

Introduction

Several halogenated alkenes, e.g., hexachlorobutadiene (HCBD), perfluoropropene (PFP), trichlorotrifluoropropene (TCTFP), and the alkyne dichloroacetylene (DCA), are selectively nephrotoxic in rats and induce proximal tubular damage (*1*). Moreover, the widely used solvents trichloroethene and tetrachloroethene, DCA, and HCBD induce carcinoma of the proximal tubules in rats. Recent studies that have sought an explanation for the selective nephrotoxicity and carcinogenicity of these compounds have elucidated a bioactivation mechanism that involves glutathione (GSH) *S*-conjugate formation, translocation to the kidney before or after metabolism to the corresponding cysteine *S*-conjugates, and, finally, metabolism by renal cysteine conjugate β-lyase to yield pyruvate, ammonia, and reactive electrophiles presumably responsible for the nephrotoxicity of the parent compounds and the cytotoxicity of the derived amino acid *S*-conjugates (*2*).

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The hypothesis that the nephrotoxicity of halogenated alkenes may be attributable to hepatic GSH *S*-conjugate formation and, ultimately, to bioactivation by renal cysteine conjugate β-lyase has been validated experimentally.

Biosynthesis of GSH *S*-Conjugates and Mercapturic Acids from Haloalkenes and Dichloroacetylene

Fluoroalkenes are reasonably good substrates for hepatic GSH *S*-transferases and are metabolized by addition of GSH to the alkene double bond (*1*). With PFP, both saturated and vinylic GSH *S*-conjugates are formed (Dekant et al., unpublished). Chloroalkenes are transformed to chlorovinylic GSH *S*-conjugates; the alkyne DCA adds GSH by enzymatic catalysis to give *S*-(1,2-dichlorovinyl) glutathione.

The reaction rates observed *in vitro* are well correlated to the chemical reactivity of the haloalkene (Table 1). In fluoroalkenes, the carbon-carbon double bond easily adds nucleophiles since it is not resonance stabilized; in TCTFP, stabilization of the intermediate carbanion, formed by reaction of the olefin with GS^- by the tri-

Table 1. Reaction rates for the GSH *S*-transferase-catalyzed conjugation of haloalkenes and dichloroacetylene with GSH in rat liver microsomes and rat liver cytosol.[a]

Haloalkene	Product formed	nmole *S*-conjugate/min/mg protein		
		Microsomes	Cytosol	Reference
Trichloroethene	*S*-(1,2-dichlorovinyl) GSH	0.002	—	(*12*)
Tetrachloroethene	*S*-(1,2,2-trichlorovinyl) GSH	0.23	0.13	(*16*)
Hexachlorobutadiene	*S*-(1,2,3,4,4-pentachlorobutadienyl) GSH	1.12	0.04	(*17*)
Perfluoropropene	*S*-(1,1,2,3,3,3-hexafluoropropyl) GSH	36	—	(*18*)
	S-(1,1,2,3,3-pentafluoropropenyl) GSH	240	136	
Trichlorotrifluoropropene	*S*-(1,2-dichloro-3,3,3-trifluoropropenyl) GSH	530	120	(*19*)
Dichloroacetylene	*S*-(1,2-dichlorovinyl) GSH	3160	660	(*20*)

[a] GSH, 10mM.

fluoromethyl substituent, increases reactivity of this chloroolefin with nucleophiles. Substitution with chlorine results in stabilization of a π-bond; chloroalkenes are more resistant to metabolism by GSH conjugation than fluoroalkenes.

With all halogenated hydrocarbon substrates studied, the extent of GSH *S*-conjugate formation is much higher with liver microsomes than with liver cytosol (*2*), a reversal of the situation observed with most other substrates for GSH *S*-transferases. This effect may be due to a preferential distribution of the highly lipophilic haloalkenes into lipid membranes, thus generating high substrate concentrations for the membrane-bound enzymes.

In rats, metabolites indicative of GSH *S*-conjugate formation have also been identified. In the bile of rats exposed to trichloroethene, tetrachloroethene, HCBD, TCTFP, or PFP, GSH *S*-conjugates identical to those formed in liver microsomes were present; in urine of exposed animals, halovinylmercapturic acids were definitively identified, demonstrating that GSH *S*-conjugate formation occurs *in vivo* (*2*).

Genotoxicity of *S*-Conjugates in Bacteria

Chlorovinyl-substituted cysteine *S*-conjugates are mutagenic in the Ames preincubation test in the absence of activating enzymes (*3*). These cysteine *S*-conjugates are converted to reactive intermediates and pyruvate by β-lyase present in the *Salmonella typhimurium* strain used. GSH *S*-conjugates derived from chlorinated alkenes require processing by γ-glutamyl transpeptidase (GGT) and dipeptidases to yield cysteine *S*-conjugates as penultimate toxic intermediates. Accordingly, the GSH *S*-conjugates derived from HCBD, trichloroethene, tetrachloroethene, TCTFP, and DCA were definite mutagens in the Ames test in the presence of rat kidney particulate fractions containing high GGT and dipeptidase activities.

To evaluate the genotoxicity of GSH *S*-conjugates and cysteine *S*-conjugates in mammalian cells, the bioactivation and detoxication mechanisms, transport properties, and DNA repair capabilities in renal proximal tubular cells were studied by using an essay to determine unscheduled DNA-synthesis (UDS) in a porcine kidney cell line (LLC PK1) (*4,5*). All halovinyl GSH and cysteine *S*-conjugate tested induce GGT and β-lyase-dependent non-*S*-phase uptake of ^{3}H-thymidin, indicative of DNA damage in LLC PK1 cells. UDS induction occurs at concentrations of *S*-conjugates that do not cause severe cytotoxicity and cell death. However, the extent of DNA repair observed was quite low and, more important, the concentration range in which UDS could be observed in the absence of cytotoxicity was very small in comparison with the effects of potent alkylating agents.

Reactive Intermediates Formed from Haloalkene-derived *S*-Conjugates by β-Lyase and Their Interaction with Nucleic Acids

β-Lyase-catalyzed cleavage of haloalkene-derived *S*-conjugates yields pyruvate, ammonia, and reactive intermediates whose structures have been recently elucidated. Two experimental approaches were developed to define the chemical nature of the intermediates formed (Fig. 1): Reactive intermediates were generated from *S*-conjugates by β-elimination and transformed to stable, characterizable products (*6,7*); and chlorinated enethiols, the initial metabolites formed from *S*-conjugates, were generated nonenzymatically in an inert solvent, and reactive intermediates were trapped and characterized.

Metabolism of halovinyl *S*-conjugates by bacterial β-lyase and by an *N*-dodecyl-pyridoxal bromide-based model system yielded halogenated acids. Reactions in the presence of diethylamine to transform intermediate acylating agents to stable products yielded the corresponding thioamides.

Cleavage of the sulfur-sulfur bond in chlorovinyl-2-nitrophenyl disulfides yields α-chlorinated enethiols (Fig. 1), as does metabolism of cysteine *S*-conjugates by β-lyase (*2*). The reduction of the sulfur-sulfur bond can be performed in inert solvents, and reactive intermediates formed can thus be studied without protein interaction or hydrolysis. To distinguish between thioacylchlorides and thioketenes as potential reactive intermediates, the ability of the carbon-sulfur double bond

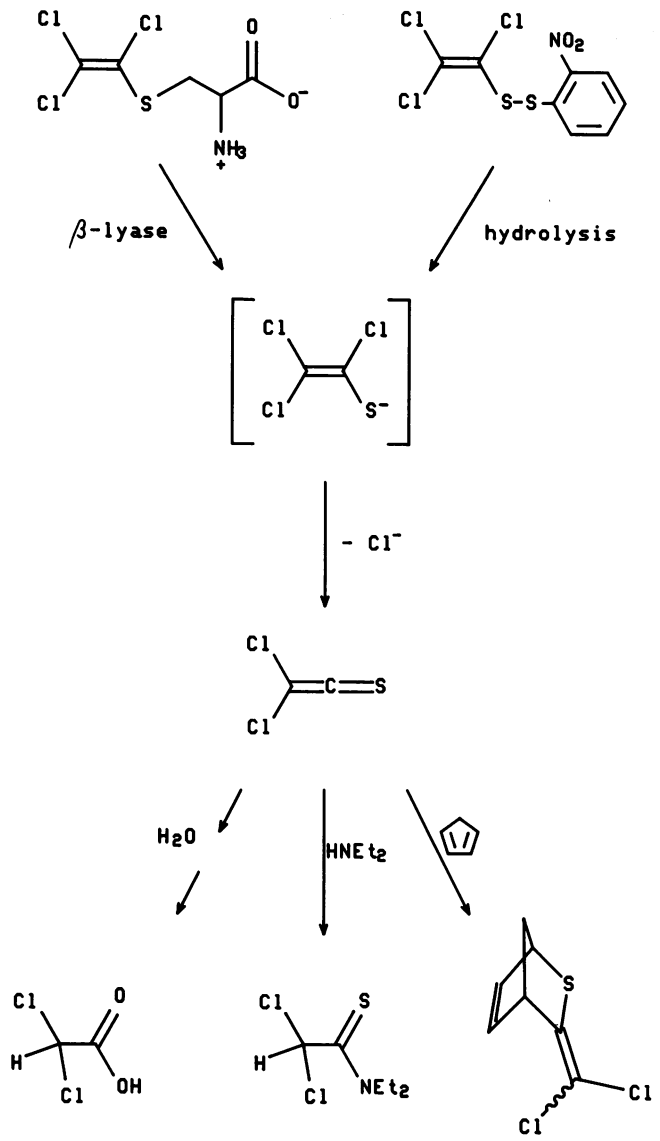

FIGURE 1. Strategy for the identification of reactive intermediates formed from cysteine *S*-conjugates by β-lyase.

to undergo Diels-Alder reactions with cyclopentadiene was used. When pentachlorobutadienyl 2-nitrophenyl disulfide was reduced in the presence of cyclopentadiene, a cyclopentadiene adduct indicative of a thioketene intermediate was identified (Fig. 1). In summary, these results suggest that thioketenes are the ultimate reactive intermediates formed by β-lyase-dependent metabolism of cysteine *S*-conjugates and that their interaction with macromolecules is responsible for *S*-conjugate induced cytotoxicity and DNA-damage.

The reactive intermediates formed from cysteine *S*-conjugates also modify DNA both *in vitro* and *in vivo*. DNA isolated from rat kidney cells and bacteria treated with ^{35}S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine (PCBC) (*8*) contained radioactivity, and when this DNA was hydrolyzed to 3′-deoxynucleotides and separated by HPLC, three distinct radioactive metabolites were detected, which probably present DNA constituents altered by PCBC metabolites.

Hydrolysis of mitochondrial DNA (*9*) obtained from the kidneys of mice treated with ^{14}C-HCBD followed by HPLC separation suggested the presence of altered DNA constituents with chromatographic properties identical with those observed with DNA constituents modified by ^{35}S-PCBC. These results demonstrate that reactive intermediates formed by β-lyase-catalyzed cleavage of cysteine *S*-conjugates interact with DNA both *in vitro* and *in vivo*.

Importance of S-Conjugate Formation and Metabolism for the Nephrocarcinogenicity of Halogenated Alkenes

The organ-specific toxicity of halogenated alkenes intensively metabolized by *S*-conjugate formation is determined by two factors: *a*) the ability of the nephron to concentrate amino acid derivatives, thus generating high concentrations of β-lyase substrates in the target cells for toxicity and carcinogenicity (*10*); *b*) the distribution of GSH *S*-conjugate processing enzymes. GSH *S*-conjugates require processing by GGT and dipeptidases prior to β-lyase-dependent bioactivation (*1*). GGT in mammals is mainly concentrated in the brush-border membrane of the proximal tubular cells. This enzyme distribution may also contribute to an increase in the concentration of cysteine *S*-conjugates in the target cells.

The importance of GSH-dependent metabolism of trichloroethene and tetrachloroethene for the nephrocarcinogenicity of the parent compounds cannot be precisely defined at present. The amounts of *S*-conjugates formed and bioactivated by β-lyase seem small and relatively insignificant (*11,12*). However, as described previously for the potent nephrotoxin HCBD, where only a small fraction of the HCBD given is bioactivated in the kidney (*13*), the contribution of the GSH *S*-conjugate/β-lyase pathway may result in nephrocarcinogenicity for trichloroethene and tetrachloroethene too, especially under the conditions of long-term bioassays with high doses. Moreover, trichloroethene inhibits its oxidative metabolism by suicidal inactivation of cytochrome P-450 (*14*); GSH conjugation reactions may, therefore, play a more important role in trichloroethene and, perhaps, also tetrachloroethene metabolism during long-term application of high doses than it does in the single dose metabolism studies carried out to date.

Trichloroethene, tetrachloroethene, and HCBD increase the incidence of renal neoplasms in rats, but only at doses that also induce severe nephrotoxicity. Tumor induction was not observed after nontoxic doses. In addition, trichloroethene and tetrachloroethene induce a low rate of renal tumors exclusively in male rats.

The formation of *S*-conjugates and their renal activation by β-lyase may be involved in this organ-specific

tumor induction. The *S*-conjugates formed are potent β-lyase-dependent mutagens in bacteria, and they induce DNA damage in mammalian cells. They are, however, also highly cytotoxic in renal proximal tubular cells. These *in vitro* results, the very low level of genotoxicity of HCBD in the kidney *in vivo* (*15*), and the severe chronic nephrotoxicity observed in long-term tumorigenicity studies indicate that both genotoxic and nongenotoxic mechanisms might contribute to haloalkene nephrocarcinogenicity. The DNA damage induced by cysteine *S*-conjugates of haloalkenes and the observed binding of HCBD-metabolites to renal DNA, however, suggests that genotoxic mechanisms are operative in the carcinogenicity of these haloalkenes.

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